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Research Article

Novel Quinone Methide Precursors: Enhanced Sensitivity and Selectivity towards Chronic Lymphocytic Leukemia (CLL) Cells

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Abstract

High progression and resistance to chemoimmunotherapies is the challenge in treatment of TP53 mutated patients. Para-nitric oxide releasing-acetylic salicylic acid (para-NO-ASA) has been shown to possess anti-neoplastic properties in CLL, but features an insufficient therapeutic index and a low efficacy against TP53 mutated CLL cells. Therefore, the para-NO-ASA was modified and the effect of these newly developed compounds *in vitro* and *in vivo* was investigated. Three of the synthesized derivatives effectively induced apoptosis with a high selectivity on CLL cells. This antineoplastic effect was independent of the TP53 mutation status. Derivative B9 demonstrated good tolerability and a strong anti-tumor efficacy in the xenograft mouse with a maximal tumor inhibition rate of 65%. Phosphorylation of the NFκB p65 subunit was significantly reduced by 10 μM B9 and B13 by around 75%, while 20 μM NO-aspirin could not induce significant reduction. The same applies to the reduction of translocation of NFκB p65 subunit to the nucleus. In addition, expressions of the NF-κB target genes BCL-2, CFLAR and BTK were clearly shut down after incubation with each substance. These results show arresting features of three newly developed derivatives making them promising compounds for high-risk CLL therapy.

Keywords: CLL; NO-ASA; TP53; NF-kappaB; Quinone Methide; Btk

1. Introduction

B cell chronic lymphocytic leukemia (CLL) is a prevalent leukemia that occurs in adults with a highly variable clinical course (Hallek, 2013). In a subset of patients with high-risk CLL, the disease progresses rapidly and requires therapy immediately. Modern treatment regimens combine alkylating agents and nucleoside analogues with monoclonal antibodies like rituximab (anti-CD20)

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and alemtuzumab (anti-CD52) (Hallek, 2013; Maddocks & Lin, 2009). Despite this state-of-the-art, CLL remains incurable. Patients relapse and become increasingly resistant to treatment. Especially mutation of TP53 and del17p are associated with poor prognosis, rapid disease progression and refractoriness towards treatment (Dal-Bo et al., 2009; Hallek et al., 2010; Rossi et al., 2009; Zenz et al., 2010a; Zenz et al., 2010b). Consequently, the need for substances which may act independently of the DNA damage pathway and/or p53, cannot be denied.

Nonsteroidal anti-inflammatory drugs (NSAIDs), renowned for application to pain, inflammation and fever, are known to possess antineoplastic properties (Robak et al., 2006; Thun et al., 2002). Not least studies have indicated that acetylic salicylic acid and analogues induce apoptosis and activation of caspases in chronic lymphocytic leukemia (Bellosillo et al., 1998; Pepper et al., 2011). Treatment with NSAIDs is always limited by side effects such as gastro-intestinal lesions. To overcome this limitation, a new substance class has been developed combining the conventional NSAID with a nitric oxide- (NO-) releasing moiety via a spacer (Brzozows et al., 2000; Fiorucci et al., 2003; Wallace & Miller, 2000). Nitric oxide plays a pivotal role in modulating the mucosal defence and is able to enhance the agreeability of the drug (Wallace & Miller, 2000). The most intensively investigated agent of this substance class, nitric-oxide donating acetylic salicylic acid (NO-ASA), has already demonstrated its tolerability in rats and humans (Fiorucci et al., 2003; Takeuchi et al., 1998).

NO-ASA showed promise to become an efficient anti-cancer drug. The anti-neoplastic effects were described for a plethora of cancer cell lines (Kashfi et al., 2002; Nath et al., 2003; Nath et al., 2005; Nath et al., 2009). Razavi et al. demonstrated a high sensitivity of primary CLL cells (IC_{50} = 8.72 μM) *in vitro* and a good inhibition of tumor growth in a chronic B cell leukemia xenograft mouse model (IR_{max} value 83.4%) for the para-isomer of NO-ASA (Razavi et al., 2011). The meta-isomer of NO-ASA tested parallel was significantly less effective *in vitro* (IC_{50} > 100 μM) as well as *in vivo* (IR_{max} value 47.9%) (Gehrke et al., 2011). Similar results were obtained by other groups working i.e. with Jurkat cells, describing the para-isomer to be considerably more potent than meta-NO-ASA (Nath et al., 2005; Rosetti et al., 2006). Due to the structural position of the NO-group, only the para-isomer is capable to generate a quinone methide after esterase bioactivation (Dunlap et al., 2007). Overall, the findings suggest a pivotal role for aromatic resonance interaction. Contrary to popular belief, neither ASA nor NO but the quinone methide contributes to cytotoxic effects (Dunlap et al., 2007; Hulsman et al., 2007). A variety of mechanisms are known to cause antitumor effects of quinones. The most commonly reported mechanism is that the quinone methide binds to cytosolic glutathione (GSH), inducing oxidative stress and leading to activation of the intrinsic apoptotic pathway (Bolton et al., 2000; Gao, Liu, & Rigas, 2005). In 2011 Williams et al. furthermore reported that NO-ASA treatment leads to nitrosation of several central messenger proteins like p53, β-catenin and p65. Most prominent is the modification p65, as it was shown to reduce NF-κB activity in various cancer cell lines (Khan et al., 2012; Williams et al., 2011; Williams et al., 2009). NF-κB can affect oncogenesis due to its ability to stimulate the expression of many antiapoptotic genes including CASP8 and CFLAR, Bcl-2, Mcl-1 and Bruton's tyrosine kinase (Btk) (Braun et al., 2006; Loop & Pahl, 1999). Many neoplasias like CLL rely on constant NF-κB pathway activity for their survival, which makes NO-ASAs ideally suited for treatment of these diseases (Furman et al., 2000). Williams et al. showed an inhibition of NF-κB activity by NO-ASA in several cancer cell lines (Khan et al., 2012; Williams et al., 2009).

Our aim is to find modifications which increase the CLL cell specific cytotoxic effect *in vitro* and *in vivo* with attention to the effects on primary CLL cells and cell lines featuring bad prognosis factors as TP53 mutation. Further, the capacity of these active agents to interfere with the aberrantly active NF-κB signalling pathway is studied.

2. Material & Methods

2.1 Patient Samples and Purification of Lymphocytes

Peripheral blood was taken from CLL patients or from healthy volunteers after informed consent. The samples were collected and processed by the (CLL-) Biobank of the Center of Integrated Oncology Cologne Bonn. The study was performed according to the World Medical Association's Declaration of Helsinki (6th version, Seoul/South Korea 2008) and authorized by the ethics committee at the University of Cologne (approval no. 04-231).

PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation. CLL cells and B cells were isolated using RosetteSep™ (Stem Cell Technology, Vancouver, Canada) according to the manufacturer's instructions. Primary cells were maintained in RPMI-1640 culture medium (PAA, Piscataway, USA) with a mix of 1 % penicillin (10,000 IU), 1 % streptomycin (10,000 μg/ml) and 10 % fetal calf serum (PAA, Piscataway, USA) at 37°C and 5% CO_2 in a humidified atmosphere.

2.2 Cell lines

Cell lines JVM-3, EHEB, U-2932, MEC-1, GRANTA-519 and SW-480 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cell lines were recovered in RPMI1640, 20% FCS, 1% penicillin/streptomycin and incubated in RPMI1640, 10% FCS, 1% penicillin/streptomycin under above-mentioned standard conditions. All experiments were carried out with fresh recovered cells obtained from and authenticated by the DSMZ, which number of cell passages is under 15. Characteristics of cell lines were listed in supplementary table 2.

2.3 Compounds and Caspase Inhibitor

Para-NO-ASA (4-((nitrooxy)methyl)phenyl 2-acetoxybenzoate), B9 (4-((nitrooxy)-methyl)phenyl benzoate), B12 (4-((chloromethyl)phenyl benzoate), B13 (4-((nitrooxy)methyl)phenyl 1-naphthoate) and analogues were provided by Prof. Dr. Albrecht Berkessel and Dipl.-Chem. Mark Krüger (Department of Chemistry, Organic Chemistry, Cologne University) (Fig. 1).

A caspase inhibitor (Z-VAD-FMK, Promega) was used to assess the role of caspases. CLL cells were incubated with 50 μmol/L of caspase inhibitor just before the addition of p-NO-ASA, B9, B12 and B13 or DMSO as a control. Cell survival was determined after 24 hours by Annexin V-FITC/PI staining.

2.4 Survival Assessment

The Annexin V-FITC/PI Apoptosis Detection Kit (BD, New Jersey, USA) was used to determine the percentage of non-apoptotic cells following the manufacturer's instructions. Annexin V/PI double-negative cells were considered non-apoptotic cells.

Caspase-Glo® 3/7 Assay and CellTiter-Glo® Luminescent Cell Viability Assays (Promega, Madison, USA) were used following the manufacturer's instructions. All calculations were done relative to 1% dimethylsulfoxide (DMSO) control.

For Annexin V-FITC/PI staining/ATP-Assay, primary CLL cells, with and without TP53 mutation, cell lines, PBMCs as well as B cells from healthy volunteers were incubated either with p-NO-ASA and the substances in a concentration range between 0.01 μM and 1000 μM, 1% dimethylsulfoxide (vehicle-control), or left untreated for 24 hours.

For Caspase-3/7-Assay, Primary CLL cells were treated either with p-NO-ASA, B9, 1% DMSO (vehicle-treated control), or left untreated for 6 hours.

2.5 Immunoblot Analysis

Analysis of proteins via immunoblotting was performed for PARP and XIAP as well as for the phosphorylation status of p65 at Ser-536, Btk and Akt.

For immunoblot analysis of the apoptosis pathway, primary CLL cells were incubated with EC_{50}'s of p-NO-ASA, m-NO-ASA, B9, B12, B13, 1% of DMSO (vehicle-treated control), or left untreated for 24 hours. For immunoblot analysis of the NF-κB pathway, primary CLL cells were incubated either with 1, 10, 20 μM p-NO-ASA, 0.1, 1, 10 μM B9, B12 and B13, 1% of DMSO (vehicle-treated control), or left untreated for 3 hours. Either whole cell lysates were extracted with mammalian protein extraction reagent (M-PER®, Thermo Scientific, Rockford, USA) supplemented with a phosphatase inhibitor cocktail (PhosphoSTOP, Roche, Mannheim, Germany) and protease inhibitor cocktail (complete Mini, Roche, Mannheim, Germany) or cytoplasmic and nuclear protein fractions were extracted using NE-PER-kit (Thermo Scientific, Rockford, USA) according to manufacturer's instructions.

Equal amounts of proteins were seperated on NuPage Novex 4% to 12% Bis-Tris gels (Invitrogen GmbH, Carlsbad, USA) and transferred to activated PVDF membranes (Immobilon®-P, Roth, Karlsruhe, Germany). Primary antibodies are listed in supplementary table 3. Secondary horseradish peroxidase (HRP)-labeled antibodies (HRP Donkey anti-rabbit IgG (Poly4064) and HRP Donkey anti-mouse IgG (Poly4053) were obtained from Biolegend (San Diego, USA).
Antibody binding was detected using Pierce® ECL Plus western blotting detection reagent (Thermo Scientific, Rockford, USA). Band intensities were analyzed using ImageJ 1.45s software (National Institute of Health, USA).

2.6 Quantitative RT-PCR

Amounts of CFLAR, BCL2, MCL1 and BTK mRNA were assessed by semi quantitative real-time reverse transcription PCR (RT-PCR) using the Light Cycler System (Roche, Mannheim, Germany).

Total RNA was extracted using RNeasy® Plus mini kit (Qiagen, Hilden, Germany) according to manufacturer's instructions after 4 h drug incubation, reverse transcribed with random hexamers and RevertAid Premium Reverse Transcriptase (Thermo Scientific, Rockford, USA). Target genes were amplified using following primers:

CFLAR: 5'- GTGGAGACCCACCTGCTCA- 3' for
5'- TTGCCTCGGCCCATGTAAT- 3' rev
BCL2: 5'- CTGTGGATGACTGAGTACCTGAAC- 3' for
5'- AACTGAGCAGAGTCTTCAGAGACAG- 3' rev
MCL1: 5'- GATTGTGACTCATTTCTTTTGGT- 3' for
5'- CTCTACATGGAAGAACTCCACAAAC- 3' rev
BTK: 5'- AATCCTCCTCCAGAAAGACAGA- 3' for
5'- GTTTTTGAGCTGGTGAATCCA- 3' rev
ABL: 5'- TGGAGATAACACTCTAAGCATAACTAAAGGT-3' for
5'- GATGTAGTTGCTTGGGACCCA-3' rev

The amount of ABL mRNA was quantified in all samples as housekeeping control and SYBR Green (Thermo Scientific, Rockford, USA) was used as the detection method. All primers were purchased from TIB MOLBIOL (Berlin, Germany). Results of real-time RT PCR were expressed as normalized target values, for example, ratio between CFLAR and ABL transcripts calculated from crossing points of each gene. All experiments were performed in duplicate.

2.7 Xenograft Nude Mice

Xenograft nude mouse model was adapted from Loisel and colleagues(Loisel et al., 2005). 1 x 10^7 JVM3 cells were injected subcutaneously into the right flank of 8 weeks old C.B.-17.Cg-Prkdc^{scid}lyst^{bg} mice. When the tumors became palpable (~ 60-80 mm^3), mice were assigned to treatment groups. The mice were treated with 8 mg /kg of B9 or 9.5 mg/kg B13 or the vehicle control (sesame oil) by intraperitoneal injection every second day in a period of 19 days. Tumor volume was approximated by the formula volume = length*(0.5*$width^2$). The percentage of the inhibition rate (IR) of the tumor volume was assessed according to the formula IR ≈ (1 - (RV_t/RV_c)) x 100, as previously described(Kawato et al., 1991).

2.8 Statistical Analysis

Statistical analyses were performed using Graph Pad Prism 5™ (Graph Pad Software, San Diego, USA) for all experiments. Data are given as mean with 95% confident intervals (CIs). Unpaired student's t-test and Mann-Whitney test were accomplished to assess statistical significance between test values and controls. P-values < 0.05 were assumed significant, and all statistical tests were two-sided.

3. Results

3.1 Newly Developed Compounds have Different Impacts on In Vitro Cytotoxicity in CLL cells

For this study, 12 new derivatives of para-NO-ASA (Fig. 1) were investigated for their ability to reduce the survival of CLL cells and healthy PBMCs (Fig. 2A).

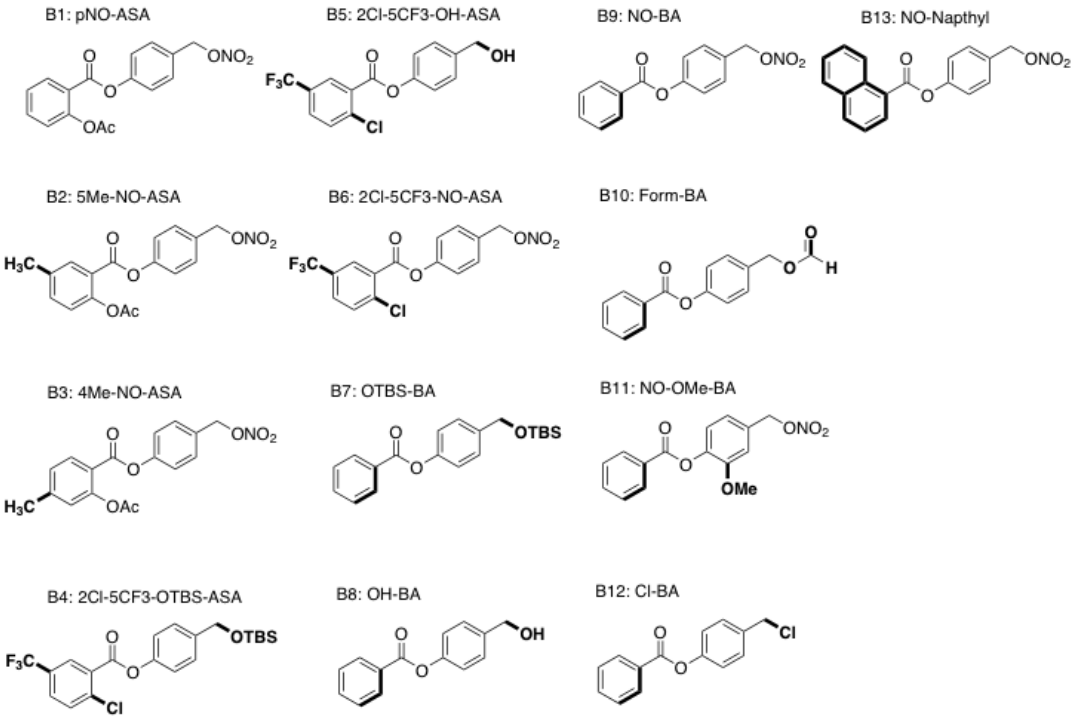

Fig. 1. The chemical modifications of nitric oxide donating acetylsalicylic acid (NO-ASA).

Six modifications of para-NO-ASA failed to act as a cytotoxic agent on CLL cells. Their median EC_{50} values are between 14.65 μM and 79.42 μM or are even out of range (Table 1A and B). As common theme, these ineffective substances can be assumed to be unable to produce quinone methide due to their chemical structure.

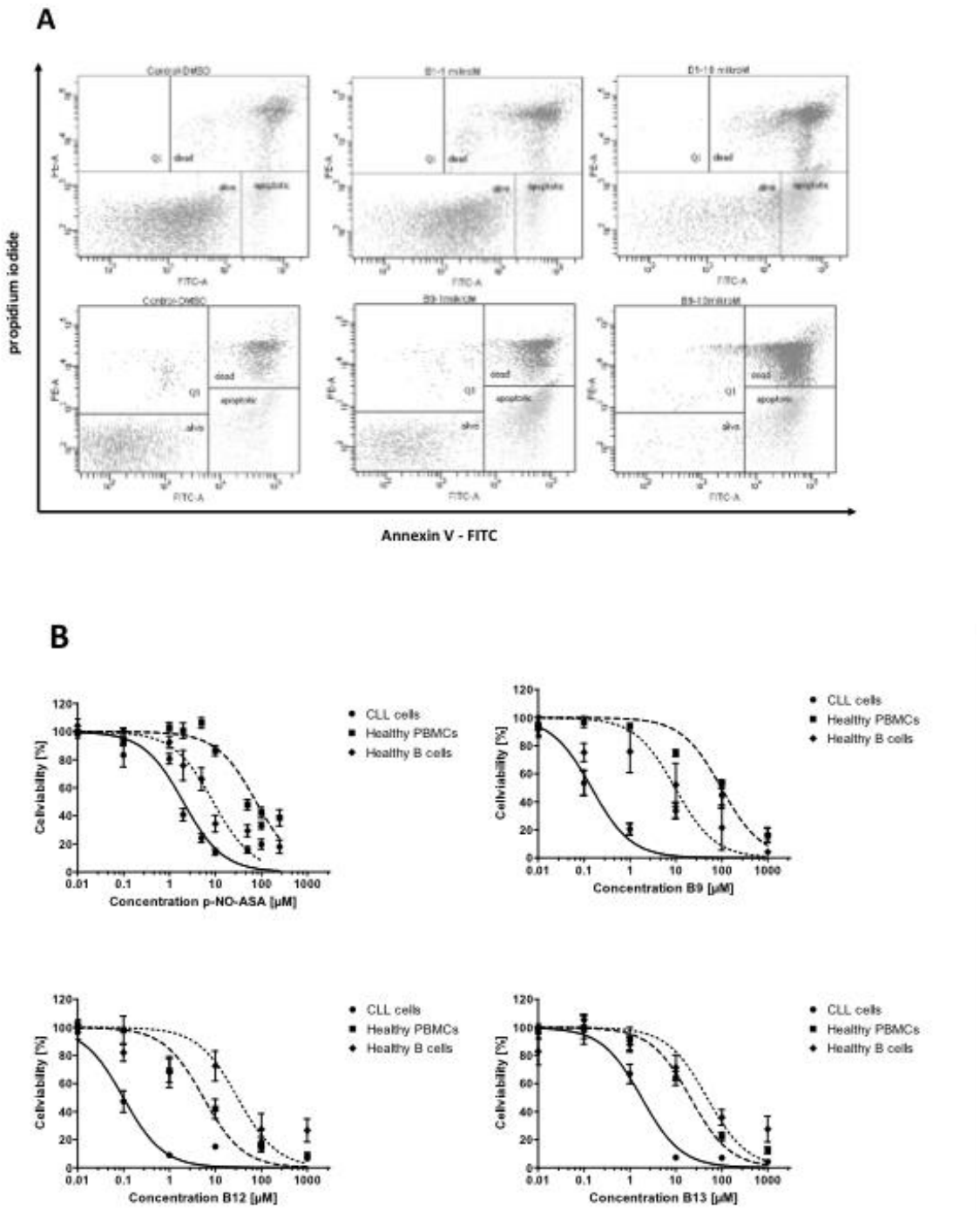

Fig. 2. Newly developed compounds have different impacts on *in vitro* cytotoxicity in CLL cells. Annexin V/propidium iodide (PI) dotblots demonstrating dose-dependent increase in

apoptosis induced by p-NO-ASA and B9 following in vitro culture for 24 h. Apoptosis was quantified by summation of the lower right quadrant and upper right quadrant (A).
CLL cells (n=10), healthy PBMCs (n=5) and healthy B-cells (n=5) were treated with different concentrations of para-NO-ASA, B9, B12 and B13 ranging from 0.01 μM to 1000 μM for 24 hours followed by addition of luminogenic CellTiter-Glo®-reagent. Para-NO-ASA, B9, B12 and B13 reduce ATP content in CLL cells more effective when compared to normal PBMCs and normal B-cells. B9, B12 and B13 give proof of a wider therapeutic margin compared to para-NO-ASA (B).

Three substances exhibit an equivalent potency and selectivity when compared to their archetype (EC_{50} B2 = 4.75 μM, EC_{50} B3 = 3.95 μM, EC_{50} B6 = 4.42 μM). All three substances carry modifications only on the ASA part of the molecule, which appears to have little influence. B9, B12 and B13 are superior to para-NO-ASA in all cytotoxicity tests. EC_{50} against CLL cells for these highly potent substances range between 1.04 - 1.84 μM. Important to note is that they are significantly less effective against PBMCs (EC_{50} ranging from 35.02 μM to 79.54 μM) compared to para-NO-ASA. These compounds carry are structurally related to their archetype para-NO-ASA. In B9, the acetoxy-group is deleted and therefore the benzene ring gets less polar. In B13, the phenyl-residue is replaced with a naphthyl-moiety also reducing the polarity. These structural modifications led to a higher cell-permeability compared to the parent compound. Exchange of the nitric-ester group to chloride affords B12.

Table 1A Compounds have different impacts on *in vitro* cytotoxicity in CLL cells (FACS-analysis). CLL cells (n=10), healthy PBMCs (n=5) were treated with different concentrations of 13 modifications ranging from 0.01 μM to 1000μM for 24 hours followed by annexin V/PI facs analysis. Mean EC_{50} values of each modification for CLL cells or PBMCs from healthy donors are shown.

No.	CLL cells EC_{50} (μM) CI 95% (μM)	Healthy PBMCs EC_{50} (μM) CI 95% (μM)
para-NO-ASA	5.582 (3.78 to 8.25)	53.89 (39.2 to 74.1)
B2	4.745 (3.04 to 7.41)	48.50 (37.1 to 63.4)
B3	3.945 (2.53 to 6.16)	> 150 (n/a)
B4	> 150 (n/a)	> 150 (n/a)
B5	37.20 (22.5 to 61.6)	96.57 (76.7 to 151.0)
B6	4.419 (1.91 to 10.2)	73.91 (40.0 to 176.5)
B7	52.78 (29.2 to 95.5)	> 150 (n/a)
B8	57.31 (34.6 to 95.0)	> 150 (n/a)
B9	1.835 (0.98 to 3.43)	79.54 (44.5 to 142.3)
B10	79.42 (48.5 to 130.2)	> 150 (n/a)
B11	14.65 (5.44 to 39.5)	20.08 (11.8 to 50.7)
B12	1.332 (0.82 to 2.15)	35.02 (12.9 to 98.3)
B13	1.043 (0.53 to 2.06)	52.70 (20.8 to 133.6)

CI = confidence intervall, EC_{50} = middle effective concentration

Table 1B Compounds have different impacts on *in vitro* cytotoxicity in CLL cells (ATP-assay). CLL cells (n=10), healthy PBMCs (n=5) and healthy B-cells (n=5) were treated with different concentrations of para-NO-ASA, B9, B12 and B13 ranging from 0.01 μM to 1000μM for 24 hours followed by addition of luminogenic CellTiter-Glo®-reagent. Para-NO-ASA, B9, B12 and B13 reduce ATP content in CLL cells more effective when compared to normal PBMCs and normal B-cells. B9, B12 and B13 give proof of a wider therapeutic margin compared to para-NO-ASA.

No.	CLL cells EC_{50} (μM) CI 95% (μM)	Healthy PBMCs EC_{50} (μM) CI 95% (μM)	Healthy B cells EC_{50} (μM) CI 95% (μM)
Para-NO-ASA	1.995 (1.49 to 2.68)	77.08 (60.2 to 98.7)	9.264 (6.72 to 12.7)
B2	1.158 (0.78 to 1.72)	53.55 (40.6 to 70.6)	5.624 (3.85 to 8.22)
B3	2.169 (1.50 to 3.15)	45.44 (28.2 to 73.5)	4.105 (2.80 to 6.03)
B9	0.767 (0.06 to 0.35)	97.13 (72.4 to 130.3)	22.88 (10.9 to 48.3)
B12	0.099 (0.06 to 0.15)	5.49 (3.23 to 9.33)	30.29 (12.7 to 72.3)
B13	1.785 (1.23 to 2.59)	20.60 (23.5 to 96.2)	47.53 (23.5 to 96.2)

CI = confidence intervall, EC_{50} = middle effective concentration

3.2 B9, B12 und B13 Selectively Decrease CLL Cell Viability

To test the selectivity the derivatives were tested on CLL cells as well as on PBMCs and B cells from healthy donors. The results show an increased therapeutic margin for B9, B12 and B13 when compared to para-NO-ASA (para-NO-ASA: $EC_{50\ CLL}$= 2.00 μM, $EC_{50\ healthy\ B\ cells}$ = 9.26 μM). For example, B9 has a superior effect against CLL cells (EC_{50} B9 = 0.77 μM) and is less effective against healthy B cells (EC_{50} B9 = 22.88 μM) (Fig. 2B). The EC_{50}'s of CLL cells and healthy B cells are given in Table 1B.

3.3 Induction of Caspase Activity in Primary CLL Cells

To investigate whether the toxicity on CLL cells is due to apoptosis, the cleavage of PARP and XIAP were investigated by immunoblot. All compounds tested induce PARP and XIAP cleavage (Fig. 3A). Para-NO-ASA and B9 also showed a concentration dependent activation of caspases 3 and 7 (EC_{50} B9 = 0.23 μM, 95% CI = 0.11 to 0.49 μM; EC_{50} para-NO-ASA = 1.84 μM, 95% CI = 0.81 to 4.21 μM) (Fig. 3B). Using the pan-caspase-inhibitor Z-VAD-FMK caused the neutralization of the cytotoxic effects of para-NO-ASA, B9, B12 and B13 in TP53 unmutated CLL cells as well as in TP53 mutated CLL cells. For example, the survival rate of 89.0% during caspase-inhibition compared with 28.9% after B9 treatment alone demonstrates the cytotoxic effect through caspase-activation (Fig. 3C).

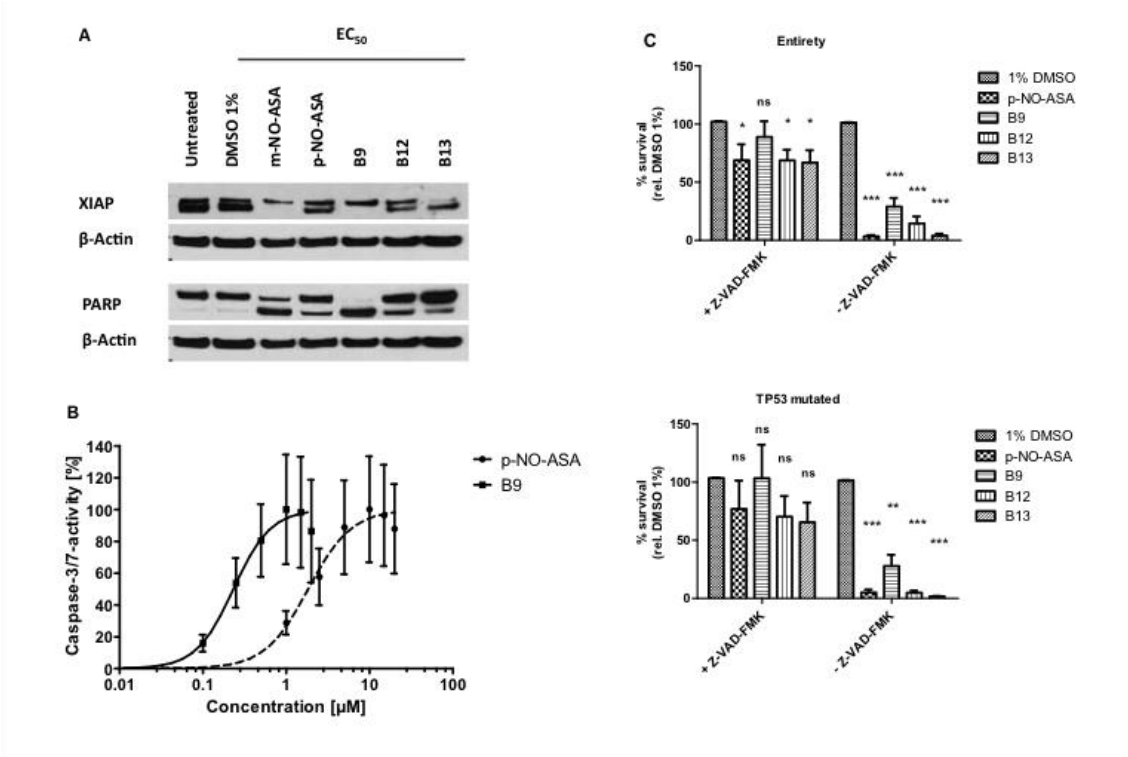

Fig. 3. para-NO-ASA, B9, B12 and B13 demonstrate their effects by inducing caspase dependent apoptotic signaling. CLL cells were cultured alone, with 1% DMSO or with EC_{50}'s of para-NO-ASA, meta-NO-ASA, B9, B12 and B13 for 24 h followed by protein lysation and westernblot analysis using antibodies to detect prognostic apoptotic proteins. Agents-treatment at EC_{50} concentration affected PARP cleavage and clearly reduced levels of anti-apoptotic proteins XIAP. Representative blots of 3 independent experiments are shown (A). CLL cells were incubated with para-NO-ASA and B9 in different concentrations ranging from 0.01 µM to 20 µM for 6 h followed by addition of luminogenic caspase-3/7-substrate. Both substances induced a concentration-dependent increase in caspase-3/7-activation (B). CLL cells with and without TP53 mutation (unmutated: n=4, mutated: n=3) were incubated with para-NO-ASA (10 µM), B9 (1 µM), B12 (1 µM) and B13 (10 µM) alone or together with the pan-caspase-inhibitor ZVAD (50 µmol/L) for 24 h. Illustrated in this figure is the survival relative to the DMSO control for a mixed group of CLL cells (TP53 unmutated as well as mutated) and for an isolated group of TP53 mutated CLL cells. For example, the survival rate of 89.0% during caspase-inhibition compared with 28.9% after B9 treatment alone demonstrates the cytotoxic effect through caspase-activation (C).

3.4 Tumor Growth is Inhibited by Treatment with B9 in CLL Xenografts

The encouraging *in vitro* results subsequently led to the issue of their efficacy *in vivo*. Due to their potentially gastro protective NO domain B9 and B13 were selected and tested for their capability to reduce and/or stabilize tumor growth in a xenograft mouse model. B9 treatment significantly reduced the tumor volume after day 9 compared to vehicle control (82.97 mm^3 for B9 vs. 134.30

mm^3 for vehicle control, p = 0.015) (Fig. 4). While the tumor volume in vehicle controls reached 594.55 mm^3 after 19 days, the B9 treated group persisted stable at 229.98 mm^3 (p = 0.0003). The maximal inhibition ratio value (IR_{max}) of B9 vs. vehicle control was 65 %.

As illustrated in Fig 4, tumor growth was less influenced by B13. The mean tumor volume of B13 treated mice increased almost parallel to the vehicle control (Day 9, 115.92 mm^3; Day 19, 537.46 mm^3).

Body weight remained stable during treatment (Supplementary Fig. 1) and we observed no severe side effects. Visual inspection of the livers, kidneys, and spleens at day 19 of five individuals from each group revealed no pathological changes (data not shown).

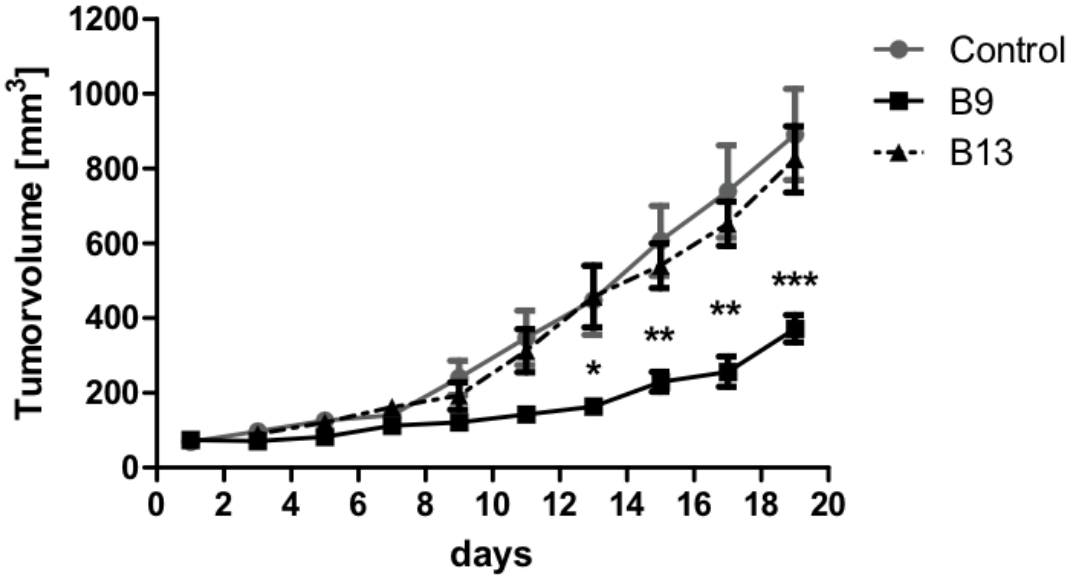

Fig. 4. B9 inhibits tumor growth in CLL xenografts, whereas B13 is less effective. Xenografted tumor harboring mice were treated daily for 19 days with 8 mg/kg body weight of B9 or 9,47 mg/kg body weight of B13 or vehicle control. B9 showed a significant inhibition of tumor growth compared to vehicle control after 9 days ($p<0.05$), increasing in significance up to day 17 of treatment ($p<0.0003$). B13 exhibited no significant inhibitory effect until day 19. The IR_{max} value was 65.33% for B9 compared to vehicle control. The error bars represent SEM.

3.5 B9, B12 and B13 Express Cytotoxicity via TP53 Independent Pathways

Based on the treatment failure of standard chemo- and chemoimmunotherapies in case of del17p and TP53 mutated patients in CLL4 and CLL8 trials, we investigated the dependency of the compounds on TP53 activity. We first analyzed the effect on survival of several cell lines either harbouring bad prognosis markers as deletion of 17p and TP53 mutation or established from

treatment resistant lymphoma cancers (Supplementary Table 2). The substances were also tested on primary CLL cells with and without TP53 mutation.

Para-NO-ASA, B9, B12 and B13 reduced ATP content in JVM-3, U2932 and EHEB cell lines significantly with EC_{50}'s from 0.29 μM to 17.92 μM. Para-NO-ASA is significantly less effective in MEC-1 and GRANTA-519 cell lines (EC_{50} = 53.44 μM and EC_{50} = 22.21 μM) compared to B9 (EC_{50} = 6.62 μM; EC_{50} = 2.28 μM), B12 (EC_{50} = 3.24 μM, EC_{50} = 0.68 μM) and B13 (EC_{50} = 24.13 μM; EC_{50} = 19.72 μM) (Fig. 5A; Table 2).

Table 2 Compounds have different impacts on *in vitro* cytotoxicity in CLL cells (ATP-assay). CLL cells (n=10), healthy PBMCs (n=5) and healthy B-cells (n=5) were treated with different concentrations of para-NO-ASA, B9, B12 and B13 ranging from 0.01 μM to 1000μM for 24 hours followed by addition of luminogenic CellTiter-Glo®-reagent. Para-NO-ASA, B9, B12 and B13 reduce ATP content in CLL cells more effective when compared to normal PBMCs and normal B-cells. B9, B12 and B13 give proof of a wider therapeutic margin compared to para-NO-ASA.

Cell line	Para-NO-ASA EC_{50} (μM) CI 95% (μM)	B9 EC_{50} (μM) CI 95% (μM)	B12 EC_{50} (μM) CI 95% (μM)	B13 EC_{50} (μM) CI 95% (μM)
GRANTA-519	53.44 (34.4 to 83.1)	6.615 (4.42 to 9.91)	3.236 (2.27 to 4.61)	24.13 (15.2 to 38.3)
MEC-1	22.21 (13.8 to 35.8)	2.279 (1.52 to 3.43)	0.679 (0.50 to 0.92)	19.72 (13.7 to 28.4)
JVM-3	3.330 (2.10 to 5.28)	2.829 (1.84 to 4.35)	1.348 (1.06 to 1.71)	17.92 (12.8 to 25.1)
EHEB	3.806 (2.96 to 4.89)	2.339 (1.62 to 3.39)	0751 (0.55 to 1.02)	13.90 (8.51 to 22.7)
U-2932	5.093 (3.22 to 8.07)	1.426 (1.07 to 1.91)	0.291 (0.22 to 0.39)	7.916 (5.12 to 12.2)

CI = confidence interval, EC_{50} = middle effective concentration

A similar picture emerges from experiments with primary CLL cells with mutated TP53. While the derivatives show a high potency against CLL cells with TP53 mutations (B9 EC_{50} = 0.69 μM, B12 EC_{50} = 0.25 μM, B13 EC_{50} = 3.20 μM), para-NO-ASA is less effective (EC_{50} = 18.37 μM). TP53 mutated CLL cells were significantly less affected by para-NO-ASA (EC_{50} = 2.00 μM, p = 0.004) (Fig. 5B).

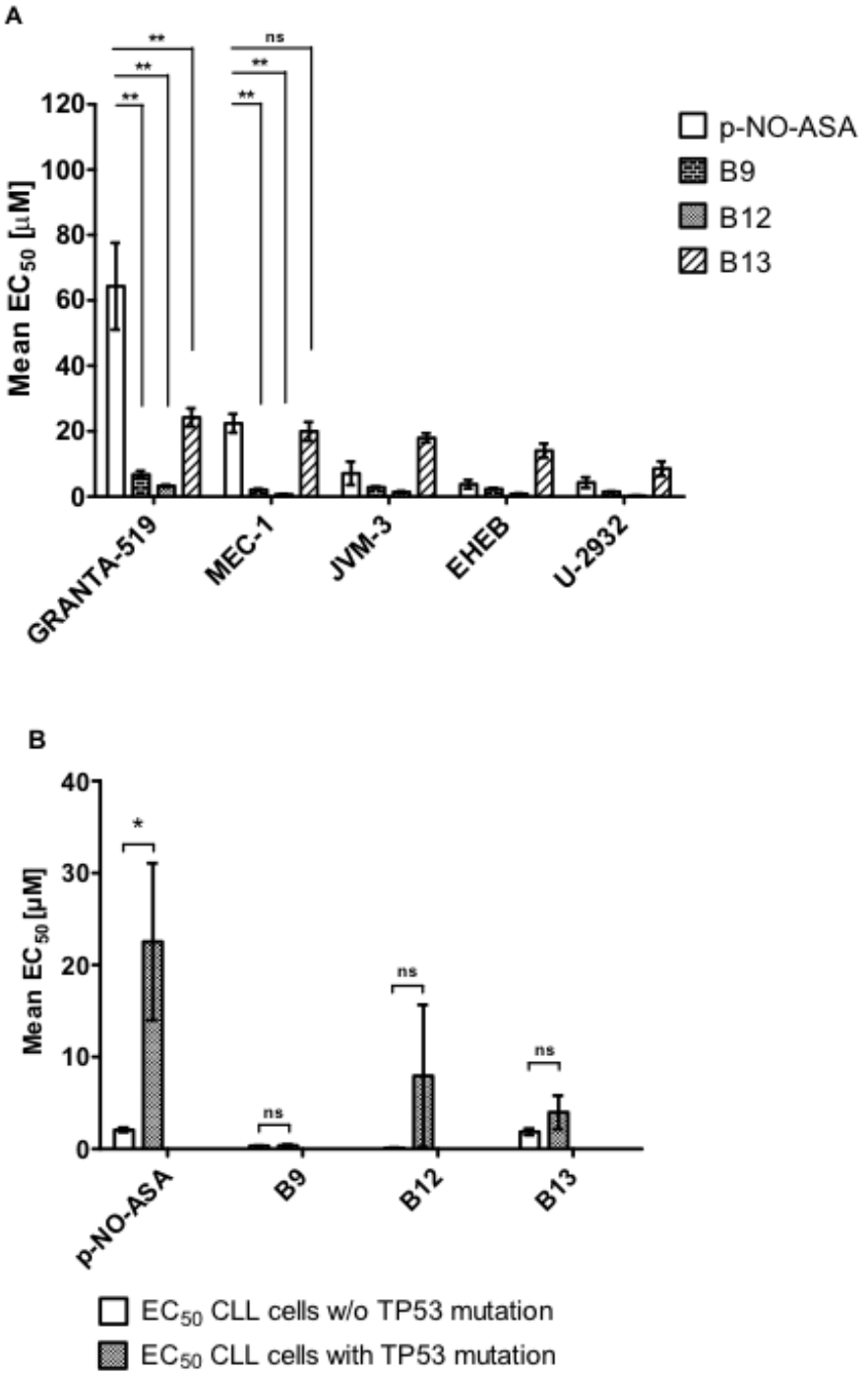

Fig. 5. Compounds B9 and B12 show superior cytotoxic effects on cell lines harboring bad prognosis factors and TP53 mutated CLL cells compared to para-NO-ASA. Several cell lines (n=5) and CLL cells with TP53 mutation (n=10) and without TP53 mutation (n=10) were

treated with different concentrations of p-NO-ASA, B9, B12 and B13 ranging between 0.01 μM and 1000 μM for 24 hours followed by addition of luminogenic CellTiter-Glo®-reagent. Para-NO-ASA, B9, B12 and B13 reduced ATP content in JVM-3, U2932 and EHEB cell lines likewise significantly, whereas para-NO-ASA is significantly less effective in MEC-1 and GRANTA-519 cell lines (A).

While the derivatives show a high potency against CLL cells with TP53 mutations, para-NO-ASA is less effective (EC_{50} = 18.37 μM). TP53 mutated CLL cells were significantly less affected by para-NO-ASA compared to TP53 unmutated CLL cells (EC_{50} = 18.37 μM vs EC_{50} = 1.995 μM, p = 0.0043) (B).

3.6 B9, B12 and B13 have a Significant Impact on NF-κB Pathway

Due to the fact that the NF-κB pathway is known to be both involved in oncogenesis and is abnormally active in CLL, we investigated the influence of the substances on this pivotal pathway. The pathway activity was assessed by examination of p65 phosphorylation levels via immunoblotting. A concentration dependent reduction of phosphorylated NF-κB p65 protein was detected upon treatment for all four substances. For 10 μM B9 and 10 μM B13, a remarkable decrease could be found (Fig. 6). Investigating the influence of these substances on p65 phosphorylation in TP53 mutated CLL cells, the above-mentioned experiment with para-NO-ASA and B9 was repeated at EC_{50} concentrations with TP53 mutated CLL cells. A reduction of phosphorylated NF-κB p65 protein was easily detectable independent of TP53 mutation status (Fig. 6B).

To gain more insight into the mechanism, we also investigated the influence of the substances on NF-κB p65 translocation into the nucleus in primary CLL cells. We detected a considerable decrease of phosphorylated NF-κB p65 protein upon 3 h treatment with all four substances (Fig. 6C).

Subsequently the impact of NO-ASA treatment on NF-κB target gene expression was analysed via RT-PCR. For this experiment, NF-κB target genes were selected which have been shown to be crucial in regulation of CLL cell viability or to be conspicuously overexpressed in CLL (Buggins et al., 2010; Kil et al., 2013; Pepper et al., 2008). Figure 6D demonstrates reduction of three NF-κB regulated genes (BCL2, CFLAR and BTK) following exposure to para-NO-ASA and compounds B9, B12 and B13. MCL1, also a classical anti-apoptotic marker, was upregulated by all four substances. For B9 and B12, we even detected a significant increase of MCL1 expression ($p < 0.05$).

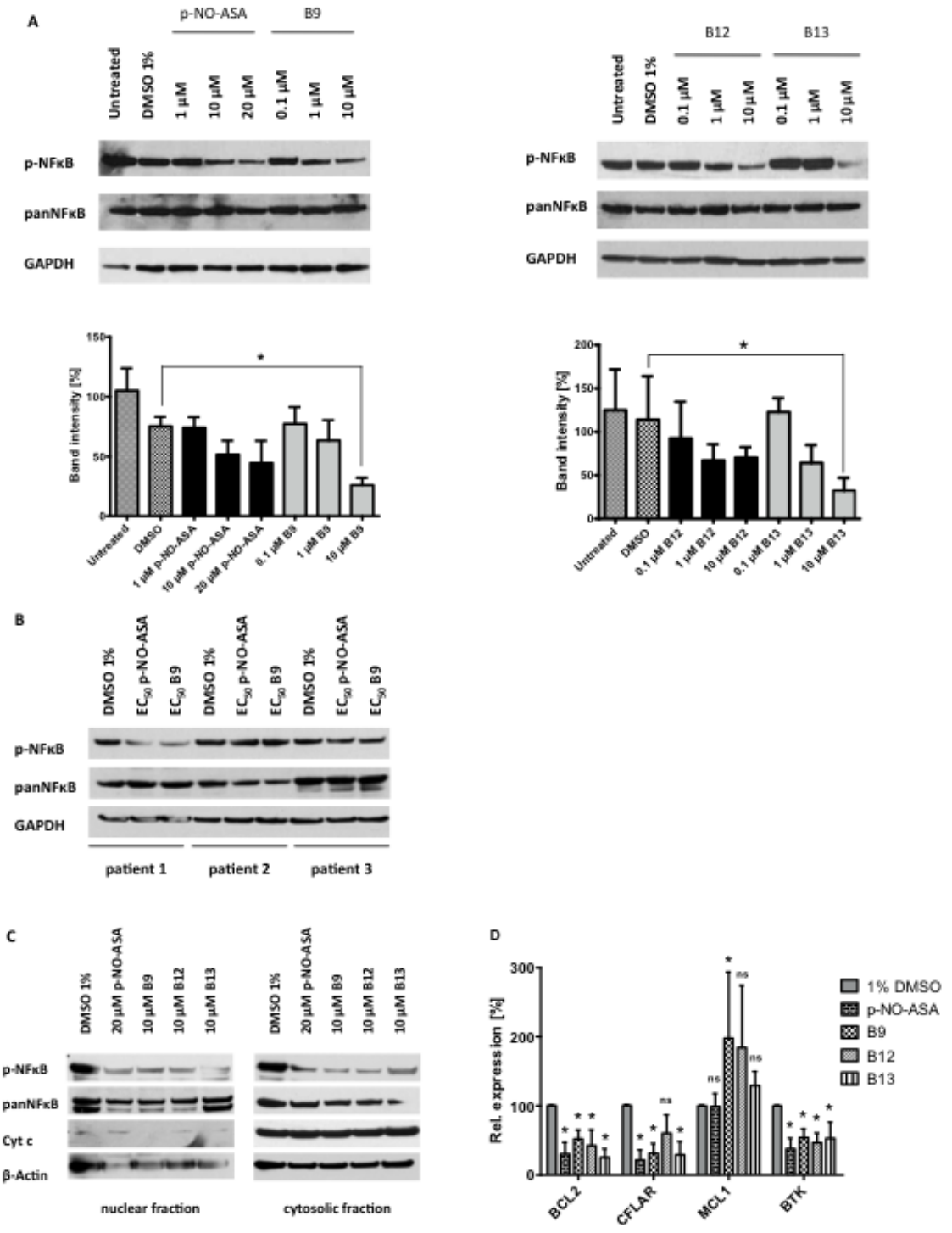

Fig. 6. Para-NO-ASA, B9, B12 and B13 inhibit NF-κB-activation and NF-κB-translocation in a concentration-dependent manner and reduce NF-κB target gene expression.

CLL cells were treated with 1, 10 and 20 μM p-NO-ASA or 0.1, 1 and 10 μM B9, B12 and B13 for 3 h. They were then harvested and lysated. Westernblot analysis revealed that phosphorylated NF-κB p65, indicating NF-κB activation, were markedly reduced with the aspirin analogues when compared to untreated controls (*$p < 0.05$). Representative blots of 3 independent experiments are shown (A).

CLL cells with TP53 mutation were treated with EC_{50} concentrations of para NO-ASA (2.00 μM) and B9 (0.77 μM) for 3 h. They were then harvested and lysated. Westernblot analysis exhibits a reduction of phosphorylated NF-κB p65 by para-NO-ASA and B9 when compared to untreated controls. Representative blot of 3 independent experiments is shown (B).

CLL cells were incubated alone, with 1% DMSO, 20 μM para-NO-ASA or 10 μM B9, B12 and B13 for 3 h. Afterwards, cytoplasmic and nuclear protein fractions were extracted. Westernblot analysis portended a decrease of phosphorylated NF-κB in the nucleus when compared to untreated controls. Representative blots of 3 independent experiments are shown (C).

We also examined transcription of NF-κB target genes by real-time PCR. CLL cells were cultured with or without 20 μM p-NO-ASA, 10 μM B9, 10 μM B12 and 10 μM B13 for 4 h. Expression of BCL2, MCL1, CFLAR and BTK were quantified using SYBR Green in real-time PCR and were normalized to housekeeping gene ABL. All four agents induced similar significant reductions in BCL2, CFLAR and BTK genes over the same time period, suggesting a common mechanism of action. Conversely, the substances markedly increase MCL1 expression. All experiments were carried out in duplicate in samples derived from 3 patients (D).

4. Discussion

In this study, we show three newly developed NO-ASA derivatives to be superior to their parent compound para-NO-ASA in experiment. These substances feature a high efficacy against CLL cells *in vitro* and, in case of B9, also *in vivo*. In addition, they are more effective against CLL cells with TP53 mutation compared to their archetype.

Para-NO-ASA has a relatively narrow therapeutic window. In comparison, B9 and B12 have higher therapeutic margins ($EC_{50\ PBMC}$ /$EC_{50\ CLL}$: p-NO-ASA = 24.1; B9 = 85.0; B12 = 187.7). These substances also exhibited lower cytotoxicity against healthy B cells ($EC_{50\ B\ cells}$/$EC_{50\ CLL}$ p-NO-ASA = 4.64; B9 = 29.83; B12 = 305.9).

The anti-neoplastic impact of the NO donating derivatives B9 and B13 was assessed in a CLL xenograft mouse model using the JVM-3 cell line. During the whole treatment period, the substances were well tolerated and revealed no obvious toxicities. B9 inhibited tumor growth in the xenograft mouse model significantly beginning with day 9. It was striking, however, that B13 showed no significant effect *in vivo*, which did not correlate with our *in vitro* findings (Fig. 4 vs. Table 1). Whether this effect is due to degradations or availability of B13 remains unclear. For

future *in vivo* studies, it will be important to optimize the drug delivery systems. Liposomal systems potentially serve as vehicles for the lipophilic compounds in CLL treatment.

A large body of literature exists, that demonstrates TP53 mutation with or without 17p deletion as poorest prognostic marker in CLL. To our knowledge, no data have been published, whether NO-ASA is effective against cancers with loss of p53 function. Therefore, we examined the ability of para-NO-ASA and its derivatives to act in a p53 independent manner. Initial tests on cell lines harbouring several characteristics revealed different potency of para-NO-ASA and its derivatives.

TP53 wildtype cell lines JVM-3, EHEB and U-2932 are highly sensitive towards treatment with para-NO-ASA and the other substances (Melo et al., 1986; Melo et al., 1988; Saltman et al., 1990). The cell lines MEC-1 and GRANTA-519 harbour a TP53 mutation and para-NO-ASA and B13 failed to effectively reduce cell viability in MEC-1 cells (Stacchini et al., 1999). B9 and B12 were effective on both cell lines. In accordance with these experiments, the sensitivity of primary CLL cells proved to be independent in TP53 status for B9, B12 and B13. In contrast, cytotoxicity of para-NO-ASA on TP53 mutated CLL cells is significantly lower than cytotoxicity on TP53 unmutated CLL cells. This indicates a TP53 independent mode of action for all tested substances but para-NO-ASA.

Several publications describe the inhibition of NF-κB as the effect of NO-ASA treatment. A reduction of phosphorylation of p65 was easily detectable for compounds presented here. Whether this effect is related to a direct nitrosation of p53 and p65, as described by Williams et al. in 2011, remains unclear (Williams et al., 2011). This is contradicted by the reduction of p65 phosphorylation by B12, which does not carry an NO-group (Fig. 6A). Therefore, it appears unlikey that p65 nitrosation is the sole reason for the inhibition of this pathway as suggested in above-mentioned study of Williams and colleagues (Williams et al., 2011). The reduction of p65 phosphorylation results in inferior transcription of NF-κB target genes including BCl-2, CFLAR and Btk. MCl-1 transcription was even significant promoted by B9. This unexpected result could be reasoned by detecting the alternatively spliced product, which promotes apoptosis. In this regard, it is important to note that other NF-κB inhibitors also failed to inhibit Mcl-1 protein expression in CLL (Pickering et al., 2007).

As mentioned above, the NF-κB target Btk was significantly reduced by para-NO-ASA and our derivatives. Btk is a signaling molecule positioned early within the BCR signaling cascade. CLL cells exhibit tonic BCR signaling activity (Scupoli & Pizzolo, 2012). Unfortunately, we could not detect any dephosphorylation of Btk or Akt, which also acts down-stream of the BCR (Supplementary Fig. 2). Nevertheless, our substances reduced the amount of Btk protein suggesting an influence on the quality of BCR signal.

In conclusion, our newly developed compounds are superior in selectivity and efficacy to para-NO-ASA on CLL cells and especially B9 is promising for CLL treatment. B9 represents a potent candidate to inhibit tumor growth *in vivo*. Whether or not derivatives without NO-donating moiety, like B12, will fulfill the expectations in *in vivo* experiments, has to be tested yet. There is evidence that the compounds described here act through inhibition of the NF-κB pathway. The experimental drugs exhibit high potency of killing TP53 mutated CLL cells; as a matter of fact, they are promising compounds for further evaluation in the treatment of CLL.

Acknowledgements

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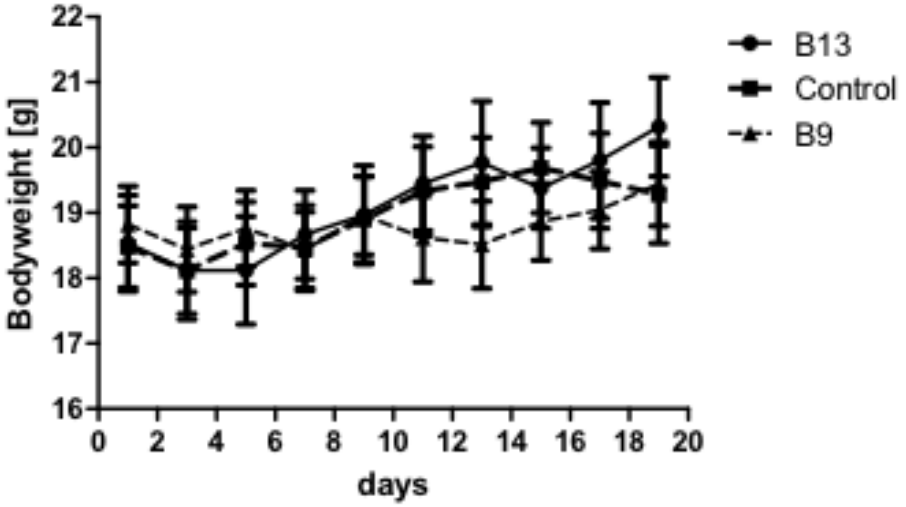
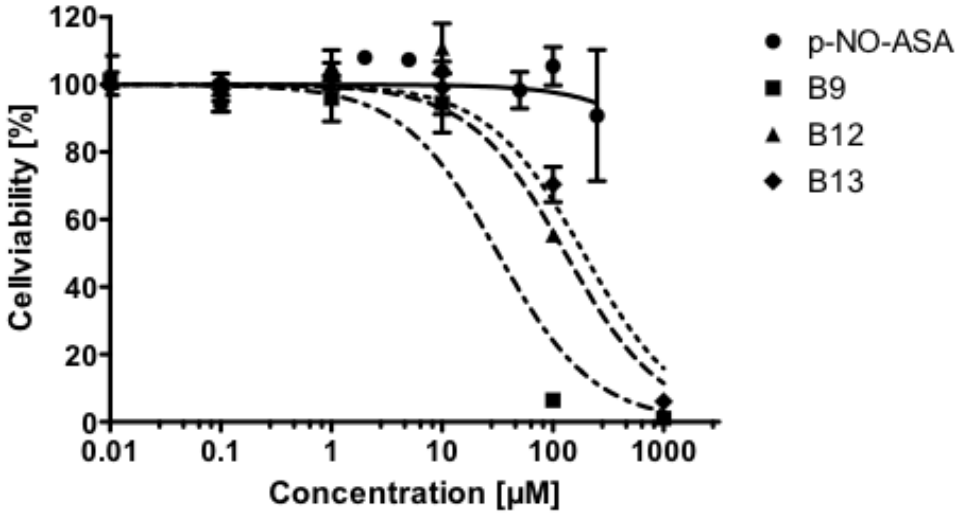
Supplementary Data

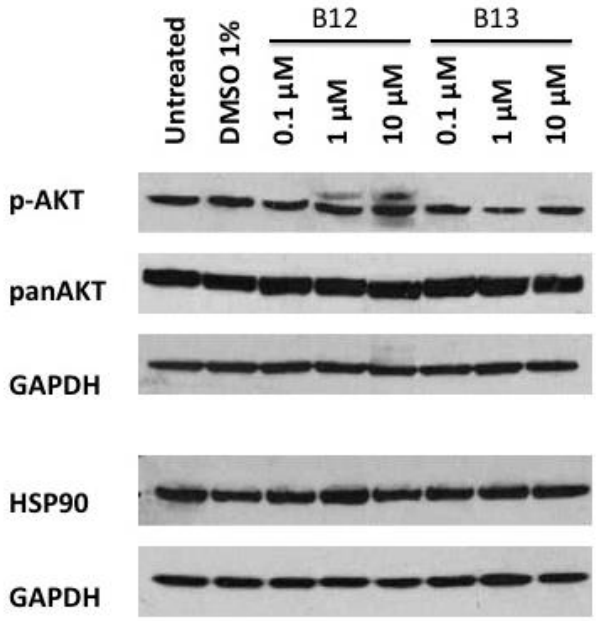


Suppl. Fig. 1. Bodyweight control of CLL xenograft mice. While daily treatment for 19 days with 8 mg/kg body weight of B9 or 9,47 mg/kg body weight B13 or vehicle-control, the body weight of the tumor harboring mice was measured every second day. There is no significant difference between body weights of vehicle control and B9 treated mice.

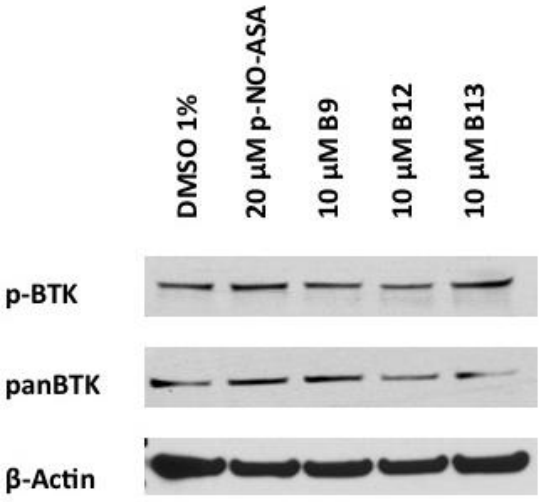


Suppl. Fig. 2. Derivatives reduce cellviability in WNT-active cell line SW480. SW480 cells were incubated alone, with 1% DMSO or different concentrations of p-NO-ASA, B9, B12 and B13 ranging between 0.01 µM and 1000 µM for 24 hours followed by addition of luminogenic CellTiter-Glo®-reagent. B9, B12 and B13 reduce ATP content significantly compared to para-NO-ASA. P-NO-ASA EC_{50} = 4251 µM ip (95% CI = 338.9 to 53319 µM), B9 EC_{50} = 31.81 µM (95% CI = 19.09 to 53.01 µM), B12 EC_{50} =

129.5 μM (95% CI = 84.02 to 199.7 μM), B13 EC_{50} = 189.5 μM (95% CI = 134.0 to 268.1 μM).



Suppl. Fig. 3. Phosphorylation of protein kinase B (AKT) is not affected by B12 and B13. CLL cells were incubated alone, with 1% DMSO, 10 μM B12 or B13 for 3 h. They were then harvested and lysated. Westernblot analysis illustrates no significant changes in phosphorylation status of AKT by B12 and B13. HSP90 protein levels are also not influenced. Representative blots of 3 independent experiments are shown.



Suppl. Fig. 4. Phosphorylation of Bruton's Tyrosinkinase (BTK) is not affected by para-NO-ASA, B9, B12 and B13. CLL cells were incubated alone, with 1% DMSO, 20 μM para-NO-ASA or 10 μM B9, B12 and B13 for 3 h. They were then harvested and lysated.

Westernblot analysis demonstrates no changes in phosphorylation status of BTK by para-NO-ASA and its modifications. Representative blots of 3 independent experiments are shown.

Suppl. Table 1 Clinical characteristics of CLL patients included in this study

Total number	67
Age [years]	
Mean	63,9
Range	49 - 85
Gender	
Male	22
Female	4
n/a	41
Binet status	
A	7
B	8
C	8
n/a	44
ZAP70 Expression	
≤ 20 %	11
> 20 %	16
n/a	40
CD38 Expression	
≤ 20 %	13
> 20 %	15
n/a	39
IgVH status	
Mutated	5
Unmutated	16
n/a	46
TP53 status	
Mutated	13
Unmutated	11
n/a	43
Del17p	
Positive	5
Negative	11
n/a	51

Suppl. Table 2. Characteristics of different cell lines*

Cell lines	Characteristics
JVM-3	B-prolymphocytic leukemia, expresses proto-oncogene BCl-2 and BCl-3, Trisomy 12
EHEB	Chronic B cell leukemia
U-2932	Treatment resistand B cell lymphoma, Overexpression of BCl-2 and BCl-6
MEC-1	Chronic B cell leukemia, del17p
GRANTA-519	Relaped high grade B-NHL. t(11;14)(q13;q32), cyclin D1 active

*Characteristics and authentication according to DSMZ online.

Suppl. Table 3. Primary antibodies

Antibody	Clone	Company	Dilution
phospho-NFκB p65 (Ser 536)	93H1	Cell Signaling Technology Massachusetts, USA	1:1000
panNFκB p65	C22B4	Cell Signaling Technology Massachusetts, USA	1:1000
phospho-Akt (Ser473)	D9E	Cell Signaling Technology Massachusetts, USA	1:1000
panAkt	C67E7	Cell Signaling Technology Massachusetts, USA	1:1000
Phospho-Btk (Tyr223)	n/a	Cell Signaling Technology Massachusetts, USA	1:1000
panBtk	D3H5	Cell Signaling Technology Massachusetts, USA	1:1000
HSP90	C45G5	Cell Signaling Technology Massachusetts, USA	1:1000
PARP	46D11	Cell Signaling Technology Massachusetts, USA	1:1000
XIAP	3B6	Cell Signaling Technology Massachusetts, USA	1:1000
GAPDH	D16H11	Cell Signaling Technology Massachusetts, USA	1:2000
β-actin	AC74	Sigma Aldrich Seelze, Germany	1:10.000
Cytochrom c	n/a	PromoCell Heidelberg, Germany	1:500